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(54) Title: SINGLE-STRAND SITE-DIRECTED MODIFICATION OF MAMMALIAN GENES IN VIVO

(57) Abstract

Single-stranded oligonucleotides are employed for site-directed modification in mammalian cells to change genes encoding proteins of interest. Desirably, a marker may be included with the gene to be able to detect the insertion site and transformation may be performed in conjunction with a plasmid having a marker, where the plasmid may be cured from the host.

* See back of page

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SINGLE-STRAND SITE-DIRECTED MODIFICATION OF MAMMALIAN GENES IN VIVO

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INTRODUCTION

Technical Field

The field of this invention is site-directed modification of genes in mammalian cells.

Background

There is a great need for genetically modified mammalian cells, so as to introduce a new phenotype, correct a mutated phenotype, or inhibit a particular gene expression. There are a large number of genetic diseases, where the mutation has been established, and the list should increase as further diseases and their etiology are investigated and determined. In many situations, particularly the hematopoietic system, there are opportunities to genetically modify cells and reintroduce the cells into the mammalian host, where the genetically modified cells may function and correct a genetic or physiological defect. Diseases such as α- and β-thalassemia, sickle-cell anemia, are immediately evident opportunities for genetic treatment.

In modifying cells, there are substantial concerns. Normally, the number of cells available may be fairly limited. Secondly, it is important that genetic modification occur at the target site and not

at other sites. Unless the modification is specific, the insertion of the introduced DNA at other sites may result in cellular modifications which could be detrimental. It is therefore important in providing for transformation of a cellular host, that the cells be transformed with high efficiency, that methods be provided which allow for identification and isolation of the modified cells, with some certainty of there being a single insertion at the desired target site. It is, therefore, of substantial interest to provide techniques and reagents which allow for enhanced efficiency in site-directed gene modification.

Relevant Literature

Site-directed gene insertion in mammalian chromosomes is described by Smithies, et al. (1985) Nature 317:230-4; Thomas, et al. (1986) Cell 44:419-28; Thomas and Capecchi (1987) Cell 51:503-12; Doetchman, et al. (1987) Nature 330:576-8; and Mansour, et al. (1988) Nature 336:348-352. The participation of single-stranded DNA in homologous recombination in mammalian cells has been reported by Rauth, et al. (1986) 83:5587-91, with as few as 25 bp of DNA sequence homology adequate for recombination (Rubnitz and Subranani (1984) Mol. Cell. Biol. 4:2253-58); Ayares, et al. (1986) Proc. Natl. Acad. Sci. (USA) 83:5199-5203. Moerschell, et al. (1988) Proc. Natl. Acad. Sci. (USA) 85:524-8 describes the modification of yeast genes using single-stranded synthetic oligonucleotides.

SUMMARY OF THE INVENTION

Site-directed modification is achieved by employing single-stranded oligonucleotide fragments having at least 40 nucleotides of homology at the site of interest, where the sequence desirably has an internal marker for detecting the presence of the desired modification.

DESCRIPTION OF THE SPECIFIC EMBODIMENTS

Methods and composition are provided for transformation with single-stranded oligonucleotides having at least 40 base homology with a target site. The transformation may be achieved under conventional transformation conditions in culture. After selection and amplification of the transformed cells, the cells may be screened. Desirably a marker is employed as part of the oligonucleotide to allow for determination of the copy number and the site of insertion of the oligonucleotide in the mammalian cell genome.

The oligonucleotides will be at least about 40 bases and not more than about 2 kb, usually not more than about 1 kb. The sequences will have substantial homology with the site of insertion, but may differ by one or more bases, usually not more than about 10, more usually not more than 5 bases from the target sequence.

The sequences will be selected to modify a particular sequence, normally to change the phenotype of the cell. Thus, the sequences may provide substitutions, both transitions and transversions, insertions and deletions, in order to change the sequence present in the host.

Sequences of interest will frequently be associated with mutations causing diseases. These sequences may be involved with the globin genes, in sickle-cell anemia, and β-thalassemia, with adenosine deaminase gene in severe combined immunodeficiency, etc. The situations where genetic modification will be desirable include sickle cell anemia and thalassemias, as well as other genetic diseases. Therefore, in the subject invention, normally the sequence of interest of the properly functioning gene, as well as the mutation will be known. However, in many instances it may not be necessary to know the specific defect, so long as one knows the region of the defect, and the sequences flanking the defect.

As already indicated, it will normally not be necessary to have complete homoduplexing in the regions where the amino acid sequence is to be conserved and in some instances it will be desirable to have one or a few mismatches. Since it will be desirable to be able to ascertain how many copies of the oligonucleotide became inserted and whether insertion was at the target site, the oligonucleotide may be designed to provide the desired amino acid sequence, while also providing for a restriction site which is not naturally present in the wild-type gene, nor in the defective gene. In this manner, transformed cells may be screened to identify the presence of fragments having homology to the oligonucleotide, where these fragments may be cleaved at the restriction site. One could then identify that the oligonucleotide had been inserted at that site.

By further showing that flanking regions had the proper sequence, one could establish whether the oligonucleotide was inserted at the appropriate site or at a different site. Thus, one could rapidly determine by employing gel electrophoresis, Southern hybridization, or other screening technique, whether one had cells in which the proper modification had occurred. Particularly, one can employ a polymerase chain reaction, using primers to sequences which would flank the oligonucleotide. In this way, a relatively large amount of DNA could be obtained, which could be sequenced or hybridized to determine whether the desired modification had occurred.

Rather than have homology to a structural gene, the oligonucleotide may have homology to a regulatory region, intron or other sequence which can affect the nature and amount of an expressed product. In this way one can modify splicing sites, inactivate or activate enhancers, promoters, inducible regulatory regions, etc.

The subject method may be used with any mammalian cells of interest, including primates, particularly humans, domestic animals, e.g. bovine, equine, feline, canine, etc.

The cells will normally be transformed in culture, usually as dispersed cells, although in some instances, tissue slices or chunks may be involved, particularly where one is not concerned with having all cells of the desired phenotype, but only having a sufficient number of cells having the desired phenotype. About 0.1 - 100 μg of DNA/10^6 cells will usually be employed. Organs which may be involved or cells from such organs include blood, bone marrow, lymph node, skin, endothelium, muscle, brain, central nervous system, thymus, liver, kidney, pancreas, etc. Specific cells may be B-cells, T-cells, neurons, glial cells, macrophages, monocytes, stem cells, retinal pigment epithelial cells, etc.

The cells will normally be present in an appropriate medium, for example, DMEM supplemented with appropriate growth factors, conveniently components of fetal serum. The cells may be transformed by any convenient technique, such as calcium phosphate DNA coprecipitates, electroporation, liposome endocytosis, microinjection, etc. The particular manner of transformation is not critical to this invention.

In some instances, it may be desirable to cotransfect with a plasmid which may be cured from the host. A temperature sensitive or inducible replication system may be used, where the plasmid may be readily cured from the host cells by maintaining the host cells at a non-permissive temperature or in the absence of the inducer. In this manner, various markers may be introduced in conjunction with the oligonucleotide sequence, so as to select for those cells which have been transformed. It is found that cells which accept DNA are likely to accept all forms of DNA present.

There is thus a high probability that if the plasmid has been accepted, the oligonucleotide will also be present in the same cells. Various markers may be present on the plasmid, particularly antibiotic resistance, e.g. G418 resistance. Various replication systems may be used, such as adenovins, papilloma virus, simian virus, Epstein-Barr virus, etc.

Once the cells have been identified as having the proper modification, these cells may be amplified and returned to the host as appropriate. Amplification can be achieved by growth in an appropriate culture medium in the presence of the proper growth factors. These cells may then be stored before administration to the host in an appropriate manner, depending upon the nature of the cells. For bone marrow, the cells may be introduced into the circulatory system by injection to provide for normal cells, hematopoietic cells may be transfused back to the patient; etc. In some instances, grafts may be involved, where tissue may be grafted onto existing tissue in the patient.

The following examples are offered by way of illustration and not by way of limitation.

EXPERIMENTAL

An oligonucleotide sequence was prepared having the following sequence:

```
                    GATC
                   C   C
                    TAGG
                     GC
                     GC
-GATGGATTGCACGCAGGTTCTCCGGCCGCTTGGGTGGAGAGGCTAT-  Neo IL
   GGATTGCACGCAGGTTCTCCGGCCGCTTGGGTCGAGAGGC       Oligo
```

The oligonucleotide is compared with the neomycin sequence with the Cla I insert. The underlined nucleotide indicates the substitution.

The complementary double-stranded DNA substrate was pSV2neoIL, which was derived by insertion of a 14 bp Cla I linker into the coding region of a neomycin phosphotransferase gene. Insertion renders the neo gene inactive. The oligonucleotide was a synthetic oligodeoxynucleotide of 40 nucleotides. It contained the wild-type sequence at the region corresponding to the insertion. In addition, it contained a single base change which is silent but creates a Taq I restriction endonuclease recognition site.

For each transfection 1 X 10^6 human EJ cells were plated in 60 mm dishes. The cells were grown in DMEM supplemented with 10% fetal bovine serum (FBS). One to 2 days later, calcium phosphate DNA coprecipitates (Lowy, et al. (1978) J. Virol. 26:291-8) were added to each plate. Each dish received 10 µg of plasmid DNA with or without an equal mass of oligonucleotide. Four hours later, the precipitate was removed and the cells treated with 20% (v/v) dimethyl sulfoxide for 2 min, rinsed and placed immediately in DMEM with 10% FBS. The next day, the cells were transferred to 100 mm dishes in DMEM containing 10% FBS and 400 µg/ml G418. Colonies were counted after 14 days.

The following table indicates the results.

TABLE 1

Subtrates	No. of Expts	Plasmid DNA used[a] (µg)	G418^R Colonies Total	per µg
pSV2neoIL + oligo	11	326	17	0.052
pSV2neoIL	3	400	1	0.0025
pSV2neoIL + heterologous oligonucleotide	2	320	0	<0.0031
pSV2neoIL (Cla I) + oligonucleotide	4	64	121	1.89
pSV2neoIL (Cla I)	4	40	22	0.55

[a] Refers only to the amount of plasmid DNA added. When included, an equal mass of oligonucleotide was present. By heterologous oligonucleotide is intended sequence heterologous to the neo gene.

pSV2neoIL (Cla I) indicates the presence of the 14 bp linker in the neo gene.

Genomic DNA was purified from G418 resistant cell lines obtained from transfection of EJ cells with pSV2neoIL and the oligonucleotide. 1 µg of DNA was used in an enzymatic amplification procedure to amplify an 800 bp fragment spanning the region of interest. The polymerase chain reaction was performed with a Perkin-Elmer Cetus DNA amplification kit. Thirty cycles of 94°, 1 min, and 65°, 5 min, were performed using an automated thermal cycler. The amplified fragment (800 bp) along with the rest of the reaction mixture was electrophoresed on a 1% agarose gel. The DNA was subsequently transferred to nitrocellulose and hybridized as described by Wood, et al. ((1985) Proc. Natl. Acad. Sci. (USA) 82:1585-8) under conditions

which allow discrimination on the basis of a single mismatch with the probe.

The amplified products from 2 of the 4 cell lines examined hybridized to the oligonucleotide indicating that in these cell lines, the oligonucleotide participated in the recombination reaction.

Plasmid DNA was rescued by fusing the $G418^R$ cell lines to monkey COS cells followed by isolation of low molecular weight DNA. Digestion of pSV2neoIL with Cla I linearizes it, producing a 5.7 kb molecule. Neither wild-type (WT) pSV2neo nor the rescued plasmid are Cla I sensitive and they migrate as a mixture of closed and nicked circles. Wild-type pSV2neo digested with Taq I yields 3 major bands, 1 of 2.1 kb, and 2 of 1.4 kb. The appropriate nucleotide substitution encoded by the oligonucleotide results in the change of a 1.4 kb band to a 1.2 kb band.

The rescued DNA was used to transform recA⁻ E. coli. The DNA contained an additional Taq I site and also contained the silent mutation as determined by DNA sequencing. Analysis of 36 plasmids recovered from different $G418^R$ cell lines derived from transfection of pSV2neoIL alone failed to reveal any molecules which contained the Taq I site. In contrast 14 out of 50 or 28% of plasmids derived from colonies in which the pSV2neoIL was used in conjunction with the oligonucleotide contained the Taq I site. Since not all of the information present on the oligonucleotide is necessary for the correction of the mutant plasmid, it is possible that a larger proportion of the plasmids are the result of homologous recombination.

Based on these results, a single-stranded DNA may provide higher efficiency of transformation at homologous sites in mammalian cells as compared to non-homologous sites than double-stranded DNA. Thus, single-stranded DNA may find preferred usage in

site-directed modification.

All publications and patent applications cited in this specification are herein incorporated by reference as if each individual publication or patent application were specifically and individually indicated to be incorporated by reference.

Although the foregoing invention has been described in some detail by way of illustration and example for purposes of clarity of understanding, it will be readily apparent to those of ordinary skill in the art in light of the teachings of this invention that certain changes and modifications may be made thereto without departing from the spirit or scope of the appended claims.

WHAT IS CLAIMED IS:

1. A method for obtaining site-directed modification in vivo at a genomic target site to change the phenotype of a cell, the modification at the target site occurring with high efficiency, said method comprising:

combining under transforming conditions a single stranded oligodeoxynucleotide with mammalian cells, wherein said oligonucleotide is substantially homologous to a target site, but differs by at least one nucleotide; and

selecting for cells comprising said oligonucleotide sequence at said target site.

2. A method according to Claim 1, wherein said transforming conditions comprise calcium phosphate precipitated DNA.

3. A method according to Claim 1, wherein said oligonucleotide sequence comprises a restriction site absent at said target site.

4. A method according to Claim 1, wherein said oligonucleotide has at least 40nt of homology with said target site.

5. A method according to Claim 4, wherein said oligonucleotide is from about 40nt to 2knt.

6. A method according to Claim 1, wherein said mammalian cell is an hematopoietic cell.

7. A method according to Claim 6, wherein said hematopoietic cell is a lymphocyte.

8. A method according to Claim 1, wherein said

mammalian cell is a retinal pigment epithelial cell.

9. A method for obtaining site-directed modification in vivo at a genomic target site to change the phenotype of a cell, the modification at the target site occurring with high efficiency, said method comprising:

combining under transforming conditions a single stranded oligodeoxynucleotide of at least 40nt with mammalian cells, wherein said oligonucleotide is substantially homologous to a target site, but differs by at least one nucleotide and comprises a restriction site absent at said target site; and

screening or selecting for cells comprising said oligonucleotide sequence at said target site by fragmenting the genome of said host cell, identifying fragments hybridizing with said oligonucleotide and identifying fragments having the target site sequence flanking said oligonucleotide sequence by means of said restriction site.

10. A method according to Claim 9, wherein said transforming conditions comprise calcium phosphate precipitated DNA.

11. A method according to Claim 9, wherein said oligonucleotide has at least about 40nt of homology with said target site.

INTERNATIONAL SEARCH REPORT

International Application No PCT/US90/02773

I. CLASSIFICATION OF SUBJECT MATTER (if several classification symbols apply, indicate all) [3]

According to International Patent Classification (IPC) or to both National Classification and IPC

IPC(5): A61K 31/70; A61K 31/585; A61K 31/07
US CL.: 514/23, 25, 460, 725

II. FIELDS SEARCHED

Minimum Documentation Searched [4]

Classification System	Classification Symbols
US CL.	514/23, 25, 460, 725

Documentation Searched other than Minimum Documentation to the Extent that such Documents are included in the Fields Searched [5]

III. DOCUMENTS CONSIDERED TO BE RELEVANT [14]

Category *	Citation of Document, [16] with indication, where appropriate, of the relevant passages [17]	Relevant to Claim No. [18]
X/Y	The American Journal of Clinical Nutrition, Volume 43, issued April 1986 (USA), A.B. Baraa et al., "Refinoyl B-glucuronice: an endogenous compound of human blood" see pages 481-485.	1 - 9
X/Y	Proceedings of the National Academy of Science, Volume 84, issued April 1987 (USA), M/H. File et al "Introduction of Differentication of Human Promyelocytic Leukemia Cell Line HL-60 by Retinoyl Glucuronide, a Biological activity metabolite of Vitamin A", see pages 2208-2212.	1 - 9
X/Y	Chemical abstracts, Volume 73, Number 14, issued 05 October 1970, (Columbus, Ohio, USA), N. Takabayoshi, et al., "Vitamin A Glucose Ether", see page 228, column 1, the abstract number 698342, Japan 7020,097, 09 July 1970.	1 - 9
X/Y	US,A, 4,457,918 (HOLICK ET AL) 03 July 1984 See the abstract, column 1 and the claims.	1 - 9
Y	US,A, 4,565,863 (BOLLAG ET AL) 21 January 1986, See the abstract, and column 3 lines 26-41.	1 - 9

* Special categories of cited documents: [15]

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IV. CERTIFICATION

Date of the Actual Completion of the International Search [2]	Date of Mailing of this International Search Report [2]
28 June 1990	12 SEP 1990
International Searching Authority [1]	**Signature of Authorized Officer [20]**
ISA.US	John W. Rollins

Form PCT/ISA/210 (second sheet) (May 1986)

III. DOCUMENTS CONSIDERED TO BE RELEVANT (CONTINUED FROM THE SECOND SHEET)

Category *	Citation of Document, [16] with indication, where appropriate, of the relevant passages [17]	Relevant to Claim No [18]
	intermolecular recombination in mammalian cells", pages 5199-5203, see the entire document.	
X	Journal of Cellular Biochemistry (New York, USA), Volume Supplement 0, Issue 13 Part E, Issued 03 April 1989, Campbell et al., "Homologous recombination involving single-stranded oligonucleotide in human cells", pages 277, abstract number WH113, see the entire abstract.	1-11

Attachment to PCT/ISA/210, Part II.
II. FIELDS SEARCHED SEARCH TERMS:

single-strand, recombination, homologous, oligonucleotide, mammalian, inventor's names